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Biotransformation of (*R*)-(+)-Limonene by the Psychrotrophic Fungus *Mortierella minutissima* in H_2O_2-Oxygenated Culture

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Summary

The aim of the present work is to investigate an integration of a microbial reaction system for the oxidation of limonene using a psychrotrophic strain with an unconventional oxygenation of the culture. The alternative method for increasing the dissolved oxygen concentration in culture media for biotransformation of *R*-(+)-limonene by *Mortierella minutissima* 01 is based on catalase-mediated decomposition of hydrogen peroxide (H_2O_2) into oxygen and water. Automated addition of H_2O_2 into the bioreactor made it possible to keep the dissolved oxygen concentration constant over a range from 5 to 100 % (±2 %). Perillyl alcohol and perillyl aldehyde were the main products of the limonene biotransformation. The amounts of perillyl alcohol produced during H_2O_2-oxygenated culture of *M. minutissima* 01 were over 2-fold higher in comparison with classical, stirred aeration. Some factors affecting the biotransformation yield were also investigated. The addition of 0.3 % methanol enhanced 1.4-fold the perillyl alcohol production by *M. minutissima* 01. A maximum yield of this product (258.1 mg/L) was observed between 2 and 3 days of cultivation in a medium containing 0.5 % substrate at 15 °C, pH=6.0. The bioconversion activity increased over 3.6-fold after optimization of some biotransformation conditions.

Key words: biotransformation, hydrogen peroxide, limonene, *Mortierella*, perillyl alcohol, psychrotrophs

Introduction

Terpenes, hydrocarbons derived from isoprene units, are the largest class of plant secondary metabolites. They are also produced by some animals. Up until now, over 30 000 natural terpenes have been identified (*1*). Terpenes and terpenoids are the primary constituents of essential oils of many types of plants and flowers. Essential oils are used widely as natural flavour additives for food, as fragrances in perfumery, and as healing agents in aromatherapy and traditional and alternative medicine. Monoterpenes are known to play an important role in chemical ecology, where they act as pollinator attractants, repellents, sex pheromones, alerting pheromones, antifungal defenses, or as part of defense secretion systems against predators (*2*).

Biotransformation of readily available monoterpene precursors, such as *R*-(+)-limonene, into their more valuable oxygenated derivatives is recognized as being of great economic potential to the food and perfume industries (*3*). Hydrocarbon limonene is an attractive starting substrate for conversion due to its abundance and low price [US$1–2 per kg; (*4*)]. *R*-(+)-limonene is the main constituent of orange and lemon peel oil, which is a by-product of the citrus processing industry produced in quantities of approx. 50 million kg per year (*5*). Interesting end products resulting from the bioconversion of

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limonene are perillyl alcohol, carvone and α-terpineol. Bulk prices for both enantiomers of carvone are in the range of US$30–60 per kg; prices for (–)- and (+)-perillyl alcohol are at least one order of magnitude higher (*6*). These terpenoids are naturally found in low quantities in many volatile oils and used extensively in cosmetics and flavouring of foods and beverages. Perillyl alcohol has anticancer activity and can play a role in antioxidant processes (*7,8*). Phase II trials to evaluate the efficacy of perillyl alcohol in the treatment of breast, pancreatic, and colorectal cancer are in progress (*9*).

A number of microorganisms have been reported to convert limonene to notable monoterpenoid compounds (*6,10–13*). In all those cases, mesophilic microorganisms were used. The drawbacks to this type of biotransformation system are cytotoxicity, excess volatility of terpenes, and low solubility of oxygen in aqueous solutions in the temperature range of 30–45 °C (*14*).

Despite their huge application potential in fundamental and applied fields (*15*), microorganisms isolated from cold environments have received little attention, especially in comparison with thermophiles. Their advantages and their ecological and economic values have not yet been sufficiently recognized. Nevertheless, in the past decade, an increasing interest of biotechnologists in these extreme organisms has been observed (*16–18*). For the first time, psychrophilic microorganisms have been found to be useful for the transformation of volatile substrates such as *R*-(+)-limonene (*19*). Cold-adapted microorganisms (psychrophilic and psychrotrophic) are distinguished from mesophiles by their ability to grow at low temperatures (>10 °C). Under such conditions, apart from other physiological characteristics, psychrophiles and psychrotrophs, though having slightly slower metabolic rates, have higher turnover numbers (k_{cat}) and catalytic efficiencies (k_{cat}/K_m) in comparison with their mesophilic counterparts (*16,20,21*).

The application of cold-adapted microorganisms offers energy-saving benefits as psychrophiles and psychrotrophs thrive in cold environments and during the winter season do not require expensive heating steps. High enzymatic activities and catalytic efficiencies in the temperature range of 0–20 °C prevent the risk of microbial contamination, shorten process time, provide increased reaction yields, accommodate a high level of stereospecificity, and minimize undesirable chemical reactions that can occur at higher temperatures (*18,20*). Moreover, at lower temperatures an increase in the dissolved oxygen (DO) fraction in culture media has been observed (*22*); the optimum fraction of DO is one of the important parameters of aerobic biotransformation processes.

Biochemical evidence indicates that these types of bioconversions are often initiated by cytochrome P-450 monooxygenase-mediated incorporation of a single atom of molecular oxygen into a substrate with the concomitant reduction of the other atom to water. Oxygen is only sparingly soluble in aqueous solutions (<10 ppm at 25 °C, 1 atm) and this limitation has been observed in diverse reactors, especially when operated at high cell density (*23*). A common method for increasing the DO fraction in culture media involves aeration with air or pure oxygen. Since the solubility of oxygen is low and the quantity of mycelia is often large, the oxygen supplied by air or even pure oxygen can be quickly depleted by the microorganisms. The critical DO fraction to determine the optimum capability of a microorganism varies and depends on the desired end product.

The present study examines the development of a microbial reaction system for the biotransformation of *R*-(+)-limonene by *M. minutissima*, a psychrotrophic fungus showing high catalase activity (*24*), using H_2O_2 as an oxygenation agent. This type of a monoterpene bioconversion system using unconventional oxygenation of culture has not been previously reported. However, the use of hydrogen peroxide to increase the dissolved oxygen concentration has been presented earlier for other metabolite biosynthesis systems (*25,26*).

This unconventional method of medium oxygenation is expected to have a great application potential, especially with regard to shear-sensitive aerobic microorganisms in the logarithmic phase of growth, when oxygen consumption is the highest. Due to the presence of hydrogen peroxide in the medium, the process of fermentation is quite resistant to microbial contamination. Another practical advantage of the described method is the essential reduction of power consumption in comparison with traditional aeration (*27*).

Materials and Methods

Chemicals

(–)-Perillic acid was purchased from Sigma, USA. (–)-Linalool (>97 %), (+)-perillyl alcohol, (+)-perillyl aldehyde, (*R*)-(+)-limonene, (–)-carveol and (–)-carvone at purity of >99 % were obtained from Fluka, Switzerland. Hydrogen peroxide (30 % in H_2O) was stored at 5 °C. All reagents and solvents were of analytical grade.

Microorganisms and media

The strain of *M. minutissima* 01 isolated from soils in Spitsbergen, Norway (lat. 77°33'N, long. 14°30'E) was used in this study (*19*). The composition of the basal medium (BM) used for the biotransformation of limonene was as follows (in g/L): malt extract 10, peptone 5, glucose 10, and yeast extract 5.

Batch operation

M. minutissima 01 was cultivated on a rotary shaker (150 rpm) at 20 °C in Erlenmeyer flasks (100 mL) containing 25 mL of BM. After 4 days of growth, 75-mL mycelial culture (obtained from 3 Erlenmeyer flasks) of *M. minutissima* 01 (about 6 g of dry mass per L) was aseptically transferred into a 250-mL reactor (6.5×18.0 cm) and incubated with different substrate concentrations with magnetic stirring (150 rpm) under controlled temperature (15 °C) and oxygen. If not stated otherwise, the biotransformations were carried out for 48 h. The dissolved oxygen fraction in the culture media was automatically controlled by a new self-made apparatus (*28*) which enabled automatic dosing of hydrogen peroxide (decomposed by catalase to oxygen and water) and oxygenation of the medium over a range from 5 to 100 % (±2 %). Conventional aeration was conducted using a

magnetic stirrer (150 rpm). The dissolved oxygen (DO) fraction of the fermentation broth was measured by an Ingold electrode (Mettler-Toledo GmbH, Germany). The value of the readings was expressed as the percentage of the initial level of saturation. If not stated otherwise, at the end of the experiment samples were withdrawn to determine the concentrations of substrate and products.

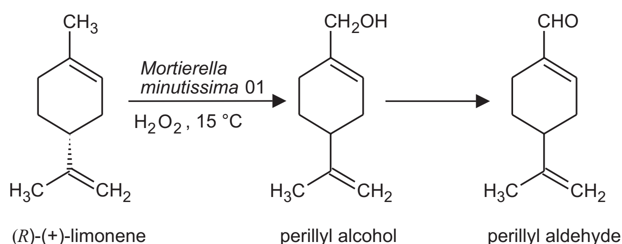
Biotransformation analysis

After the specified bioconversion time, 400 μL of an internal standard (IS) solution of 1 % linalool in hexane were added to the medium. Subsequently, the biomass was harvested by centrifugation and the liquid was extracted twice by an equal volume of diethyl ether in a separatory funnel. The ether fraction was separated, dried over anhydrous Na_2SO_4, and concentrated to dryness on rotary vacuum evaporators at a water bath temperature of 30 °C. The residues obtained were dissolved in 2 mL of hexane and used for GC and GC-MS analyses conducted according to the previously reported method (*19*). Quantification of the volatile compounds was done by comparison with the added standard. The concentrations of perillyl alcohol and limonene in each extract were quantified from individual calibration curves using peak area ratios (analyte/IS) *vs.* amount ratios (analyte/IS) from standard authentic samples. Biotransformations were performed in two replicate samples and the analyses were carried out in duplicate. The error associated with the GC quantification of samples was ±4 % and is quoted for a confidence interval of 96 %. The data given here are the average values of measurements.

Results and Discussion

Biotransformation of terpenes by bacteria and fungi is known to be markedly influenced by the DO fraction in the liquid phase (*29–31*). Oxygen has been shown to accelerate this bioconversion, but excess O_2 brought a decrease in yield (*32*). When air was supplied, bioconversion of carveol to carvone increased 10-fold as compared to the control sample, where no air was forced into the flasks (*30*).

The addition of hydrogen peroxide (H_2O_2) was elaborated as an alternative method for increasing the DO fraction in culture media and enhancing limonene biotransformation by *M. minutissima* 01, which possesses catalase activity. The main products of this process were perillyl alcohol and perillyl aldehyde (Scheme 1).



Scheme 1. Products of the biotransformation of *R*-(+)-limonene by *M. minutissima* 01 under unconventional oxygenation of the culture medium

During hydroxylation of limonene under traditional stirred aeration, the relative fraction of DO in the biotransformation system oscillated between 2–5 %. After an addition of 100 μL of 1 % hydrogen peroxide to the culture medium, DO reached 100 % saturation and then dropped within 40 min to a 5 % level.

As follows from the results presented in Table 1, the bioconversion activity of *M. minutissima* 01 was 104.9 mg/L of perillyl alcohol produced in 48 h after a single addition of 1 % of hydrogen peroxide (1 mL). A comparison of the production of perillyl alcohol by oxygenation with H_2O_2 and stirred aeration of the *M. minutissima* mycelium showed that the H_2O_2-oxygenated culture was about 1.5-fold more efficient than the culture aerated by stirring.

It has been reported that most cytochrome P-450-dependent monooxygenases can utilize hydrogen peroxide as a source of oxygen atoms for substrate monooxygenation (a process termed 'peroxide shunt'). This 'peroxygenase' activity offers an opportunity to utilize cell-free cytochrome P-450-dependent catalysis without a need for NAD(P)H regeneration, additional proteins or dioxygen (*33*). Still, it remains unclear whether this process took place in our system since the involvement of cytochrome P-450 in the conversion of limonene in the cells of *M. minutissima* 01 is yet to be confirmed.

In a subsequent experiment, various H_2O_2 fractions (1–20 %) were examined to investigate the relationship between the level of H_2O_2 and the concentration of perillyl alcohol in the medium. The results show that the yield of perillyl alcohol decreased with an increase in H_2O_2 fraction, from 180.2 (at 1 % H_2O_2) down to 129.2 mg/L (at 20 % H_2O_2) (Table 1). This was probably caused by an inhibiting effect of H_2O_2 on the mycelium of *M. minutissima* 01. Therefore, in further experiments, the 1 % fraction of H_2O_2 was used.

In order to minimize the toxic effect of limonene on the mycelium of *M. minutissima* 01, the substrate concentration for its enhanced biotransformation under unconventional oxygenation of the culture was investigated. The optimum amount of limonene (0.8 %), determined earlier by Trytek and Fiedurek (*19*), could be too high combined with the presence of hydrogen peroxide in the medium. It was found that under the tested conditions a maximum yield of perillyl alcohol (209.5 mg/L) occurred in a medium containing 0.5 % of the substrate (Fig. 1).

Cold-active enzymes can also find application in mixed aqueous–organic or non-aqueous solvents for the purpose of organic synthesis. Their utility derives from their inherent flexibility, which counteracts the stabilizing effects of low water activity in organic solvents (*18,20*). In subsequent experiments, different organic solvents with various log *P* values were tested for their ability to enhance limonene bioconversion. Only methanol (0.5 %), which is often used to improve the reaction rate in steroid bioconversion (*34*), enhanced the bioconversion by the mycelium of *M. minutissima* 01 over 1.25-fold. The optimal fraction of methanol for *R*-(+)-limonene conversion was 0.3 % (Table 1). The inhibiting effect of the solvent on oxygenation of limonene was observed when the methanol fraction was over 25 %. Tan and Day (*32*) in-

Table 1. Some factors affecting bioconversion activity of *Mortierella minutissima* 01 during oxygenation of medium by hydrogen peroxide

Varied factor	γ(perillyl alcohol) mg/L	γ(perillyl aldehyde) mg/L	γ(remaining limonene) mg/L	γ(dry mass) g/L
Control[a]	71.5	7.9	7.1	6.8
Oxygenation with 1 % H_2O_2[b]	104.9	9.8	45.1	6.8
H_2O_2[c]/%				
1	180.2	15.0	510.0	7.0
2	153.1	17.8	1132.6	7.6
4	157.5	26.7	1357.5	8.2
10	176.5	23.8	1187.8	7.4
20	129.2	16.0	483.9	6.8
Solvent[d] (0.5 %)				
Glycerol	84.1	1.3	1.2	8.0
Ethanol	57.9	4.6	68.5	6.6
Methanol	218.8	6.7	3.5	5.6
Dodecane	0.7	10.3	1836.6	7.4
Hexadecane	1.1	16.2	1957.9	7.2
Methanol[e]/%				
0.1	164.9	5.3	3.0	6.2
0.2	225.3	10.9	43.7	6.4
0.3	227.9	8.9	88.2	6.2
0.4	201.1	10.0	272.9	6.4
1.0	161.5	20.6	9.5	5.6
25.0	0	0	5.1	7.6
50.0	0	0	9.1	8.8
Conversion time[f]/h				
24	139.3	12.1	1941.3	9.2
48	258.0	11.7	109.7	7.6
72	228.3	23.5	6.9	6.8
96	207.6	6.2	2.8	6.2

Incubation conditions:
[a]The initial concentration of limonene: 6.7 g/L (0.8 %), 250 μL of distilled H_2O added at one time, temperature 15 °C, time 48 h, agitation speed 150 rpm
[b]The initial concentration of limonene: 6.7 g/L (0.8 %), 250 μL of H_2O_2 (1 %) added at one time, temperature 15 °C, time 48 h, agitation speed 150 rpm
[c]The initial concentration of limonene: 6.7 g/L (0.8 %), 250 μL of H_2O_2 (1–20 %) added after every 8 h, temperature 15 °C, time 72 h, agitation speed 150 rpm
[d]The initial concentration of limonene: 4.2 g/L (0.5 %), 250 μL of H_2O_2 (1 %) added after every 8 h, temperature 15 °C, time 72 h, agitation speed 150 rpm, solvent (0.5 %)
[e]The initial concentration of limonene: 4.2 g/L (0.5 %), 250 μL of H_2O_2 (1 %) added after every 8 h, temperature 15 °C, time 72 h, agitation speed 150 rpm, methanol (0.1–50.0 %)
[f]The initial concentration of limonene: 4.2 g/L (0.5 %), 250 μL of H_2O_2 (1 %) added after every 8 h, temperature 15 °C, time 24–96 h, agitation speed 150 rpm, methanol (0.3 %)

vestigated the effect of an organic solvent on *Penicillium digitatum* bioconversion of (*R*)-(+)-limonene to (*R*)-(+)-α-terpineol. They found that ethanol caused the inhibition of the bioconversion at a fraction of 2 %. Methanol, on the other hand, was shown to positively influence the bioconversion, at an unoptimal fraction of 0.5 %, but had a cytotoxic effect at fractions higher than 2 %. The results obtained in the present study show that methanol has a positive effect on bioconversion of limonene when applied at fractions in the range of 0.1–0.5 %. A sharp drop in the biotransformation yield, on the other hand, was observed for dodecane and hexadecane, when only a trace amount of perillyl alcohol was obtained (Table 1). Such results were probably due to the fact that the mycelium of the fungus had a limited access to the substrate inside the most hydrophobic solvents. This can also have been connected with a low mass transfer coefficient between water and these solvents. On the other hand, an increase in the production of perillyl aldehyde was observed in the presence of strong hydrophobic solvents such as dodecane, hexadecane, and limonene (Fig. 1, Table 1). This may have been caused by shifting the point of equilibrium towards the side of perillyl aldehyde under hydrophobic conditions. There is no possibility of limiting the oxygen supply in those cases since hydrocarbons can serve as oxygen vectors. In consequence, a possible anaerobic oxidation of perillyl alcohol to perillyl aldehyde could not be favoured.

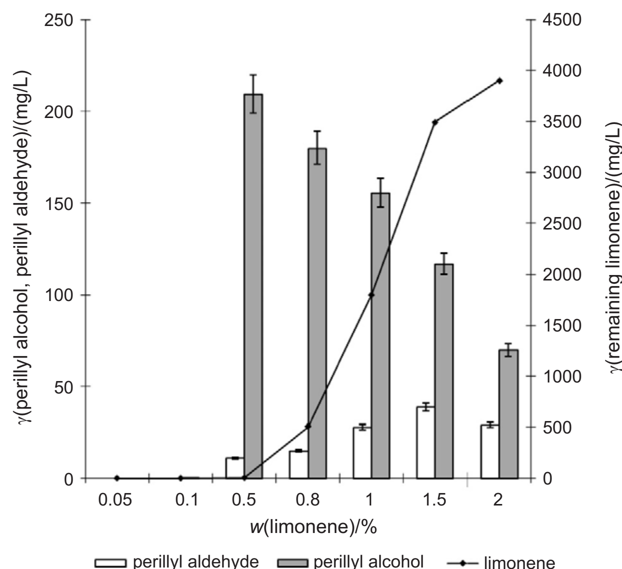


Fig. 1. Effect of substrate concentration on *R*-(+)-limonene bioconversion by *M. minutissima* 01 under unconventional oxygenation of the culture. Biotransformation conditions: no solvents, 250 μL of H_2O_2 (1 %) added after every 8 h, temperature 15 °C, time 72 h, and agitation speed 150 rpm. The bars indicate the standard errors from two replicates

The time course of bioconversion of limonene by *M. minutissima* 01 was studied by analyzing the reaction products at 24, 48, 72 and 96 h and is shown in Table 1. Gradual utilization of limonene by the fungus was observed over the bioconversion period, and the yields of perillyl alcohol increased significantly between 24 and 48 h. A similar conversion time for biotransformation of (*R*)-(+)-limonene to (*R*)-(+)-α-terpineol by *P. digitatum* was observed by Tan *et al*. (*29*). The time of limonene bioconversion by *M. minutissima* 01 was shorter than that of the same conversion conducted with *Pseudomonas putida*, which was reported to be 120 h (*10*).

The small increase in the concentration of the determined products accompanying the depletion of substrate may be caused by a loss of substrate through evaporation, and/or further metabolization of perillyl alcohol. When control experiments were run with 50 or 125 μL of limonene, dissolved in 25 mL of sterile culture broth, it was shown that the fraction of limonene dropped to 20 and 34 % of its original fraction, respectively, after 48 h. In the case of perillyl alcohol, a slight drop (8 %) in its initial fraction was noted (data not shown). Accordingly, only trace amounts of limonene were left in the cultures of *Penicillium digitatum* NRRL 1202 after 48 h of biotransformation, as observed by Tan *et al*. (*29*). This might also be a result of limonene accumulation in the mycelium. After 2 days of transformation, as observed by Onken and Berger (*35*), only 3 % of the recovered substrate were found in the culture medium, while 97 % were accumulated in the mycelium.

A comparison of the bioconversion activity of the H_2O_2-oxygenated and the stirred aerated mycelium of *M. minutissima* 01 showed the former type of oxygenation of the culture to be 2.2-fold more efficient than the latter (Table 2). This was probably due to *in situ* oxygen generation through decomposition of hydrogen peroxide by the catalase available in the periplasmic space, where it was immediately utilized by monooxygenases for the oxidation of *R*-(+)-limonene, which in all probability occurred intracellularly. In a traditionally aerated culture, efficient oxygen supply is difficult due to low oxygen solubility and high gas-liquid mass transfer resistance. Consequently, the amounts of perillyl alcohol produced during H_2O_2-oxygenated culture of *M. minutissima* 01 were high (over 2-fold in comparison with classical aeration) (Table 2).

Conclusions

The application of H_2O_2-oxygenated culture for the biotransformation of terpenes has not been described so far. It was used for the first time for limonene biotransformation by the psychrotrophic fungus *M. minutissima* 01. The new integrated oxygen supply and low-temperature bioconversion method for monohydroxylation of limonene eliminates to a considerable degree the gas-liquid transport resistance and increases the amount of available oxygen (*25*). The possibility of using 'environmentally clean' oxidants and co-catalysts such as hydrogen peroxide and methyl alcohol makes research in this area even more interesting. The results provide valuable information and references for the optimization of some process conditions. The use of the method resulted in 2-fold increased productivity as compared to classical aeration and enabled precise monitoring of oxygen consumption. In this system, 258.1 mg/L of perillyl alcohol were obtained, partly owing to the reduction of the loss of terpenes by evaporation. The combined use of the un-

Table 2. Dependence of limonene bioconversion by *M. minutissima* 01 on the level of oxygen fraction in the culture

Trial	Dissolved oxygen fraction	γ(perillyl alcohol)	Mass of perillyl alcohol produced by 1 g of mycelium dry mass	γ(mycelium dry mass)
	%	mg/L	mg	g/L
I	5	107.4	19.9	5.4
II	50	234.4	46.0	5.1
III	100	201.2	41.1	4.9

Bioconversion conditions: temperature 15 °C, limonene concentration 4.2 g/L (0.5 %), methanol fraction 0.3 %, medium volume 75 mL. Low amounts of hydrogen peroxide (2 %) were automatically added to maintain required oxygen fraction. The relative value of the dissolved oxygen fraction oscillated between 45–60 % (trial II) and 95–120 % (trial III), I: the trial to which sterile water was dispensed in an amount which was equivalent to the volume of hydrogen peroxide consumed in trial II

conventional method of medium oxygenation and psychrotrophic microorganisms showing catalase activity offers an effective strategy for the biotransformation of volatile terpenes.

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